Identification of potential protective antigens of *Ostertagia ostertagi* with local antibody probes

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SUMMARY

The identification of protective helminth antigens remains the most important challenge in the development of parasitic vaccines. To identify protective antigens of *Ostertagia ostertagi*, an important abomasal parasite of cattle, parasite-specific local antibodies from the abomasal mucus and from the draining lymph nodes were collected from calves immunized with multiple infections and from 'primary infected' animals. With these probes, Western blots of extracts and excretion/secretion (E/S) material from L_3, L_4 and adult life-stages as well as cDNA expression libraries were screened to identify antigens that were exclusively recognized by antibodies from 'immunized' calves. In the adult stage, a protein of 32 kDa was specifically detected on Western blot by mucus antibodies from 'immunized' animals. In the L_3 and L_4 larval stages, proteins situated in the regions of 28–29 kDa were recognized by mucus antibodies and a 59 kDa antigen was specifically recognized by lymph node antibodies from 'immunized' animals. Screening E/S material revealed no specific difference in recognition pattern between 'immunized' and 'primary infected' animals. Screening of the cDNA libraries revealed 26 relevant clones, coding for 15 proteins, among these several with potential protective capacity, immunodominant properties or functional and physiological importance e.g. metalloproteases, an aspartyl protease inhibitor and collagen.

Key words: *Ostertagia ostertagi*, local antibodies, protective antigens.

INTRODUCTION

Ostertagia ostertagi is the most important gastrointestinal nematode of cattle in temperate regions of the world (Vercruysse & Claerebout, 2001). At present, the control of *O. ostertagi* and other gastrointestinal nematodes is mainly based on the use of anthelmintic treatment programmes. However, anthelmintics have several drawbacks such as the threat of anthelmintic resistance and the increasing consumer awareness of the possible consequences of residual chemical substances. The development of a vaccine to control gastrointestinal nematodes should be a promising alternative (Knox, 2000).

The biggest problem in the development of a vaccine is the identification of protective antigens. The search for gastrointestinal nematode vaccine candidates has so far been based largely on immunoscreening with sera from infected animals. Western blots of protein extracts or E/S material from different parasites (McGillivery *et al.* 1992; Schallig, Van Leeuwen & Hendrikx, 1994; Schallig, Van Leeuwen & Cornelissen, 1997) as well as cDNA expression libraries (Rehman & Jasmer, 1998; Newlands *et al.* 1999) have been screened with polyspecific sera. Although serum antibodies are frequently used to screen for candidate nematode antigens, specific tissue-restricted antibody responses are not always detectable in serum. In addition, antibody responses in the lymph nodes are only detected when the parasite is still present in the tissue, while parasite-specific serum antibodies can remain high after infection and cure, suggesting that serum antibodies are maintained by antibody-secreting cells (ASC) residing outside the lymph nodes (Meeusen & Brandon, 1994*a*). Also, the persistence of serum antibodies makes it difficult to differentiate between previous and recent exposures to a pathogen. In contrast, local antibodies are likely to be more specific for antigens present in the infected tissue at the time of examination (Miller, 1987; Meeusen & Brandon, 1994*a*, *b*; Meeusen, 1996). Screening antigens with ASC antibodies has already led to the identification of a surface antigen of the infective L_3 larval stage of *Haemonchus contortus* (*Hc*-sL3) (Ashman *et al.* 1995; Raleigh & Meeusen, 1996) that showed significant protective capacity (Jacobs *et al.* 1999).

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Not only the draining lymph nodes but also the covering mucus layer from the abomasum are a source of local antibodies (Miller, 1987). In sheep infected with *Teladorsagia circumcincta*, the recognition of specific worm antigens by antibodies from the abomasal mucus seems to be part of the mechanism that regulates the fecundity of the parasite (Stear *et al.* 1995; Strain & Stear, 1999). After challenge infection of calves with *O. ostertagi*, a negative correlation between fecundity of the worm and parasite-specific IgA in the mucus was observed (Claerebout *et al.* 1999).

In this paper, we describe the results of screening Western blots from the L_3, L_4 and adult life-stages of *O. ostertagi* extract and E/S material with local antibodies from mucus and ASC culture supernatant to identify antigens that were only recognized by antibodies from 'immunized' animals and not by antibodies from 'primary infected' animals. cDNA libraries of the 3 different parasitic stages were screened with the same antibody probes to identify the nucleotide sequences that code for these antigens.

MATERIALS AND METHODS

Animals

A total of 17 calves, male and female Holstein-cross breed, between 6 and 12 months old from 3 different farms received a natural infection with gastrointestinal nematodes during a first grazing season of at least 6 months.

To confirm the immune status of the calves, reductions in worm burdens were measured after treatment with benzimidazoles at housing and at subsequent challenge infection. Calves of farm 1 ($n = 4$) received a natural challenge during 3 weeks in the second grazing season (Claerebout *et al.* 1998). Calves of farm 2 ($n = 6$) and 3 ($n = 7$) received an experimental challenge with 50000 *O. ostertagi* L3 larvae, 1 week after treatment. The *O. ostertagi* worm counts of these animals ('immunized' animals) were compared with those from helminth-free calves ($n = 6$ for each farm), which received the same challenge ('primary infected' animals). Reductions in worm counts were 48, 45 and 24% for the immunized calves of farms 1, 2 and 3 respectively, compared to the primary infected animals.

Sample collection

Mucus collection. Abomasal mucus from all 17 'immunized' animals from the 3 different farms and from the 18 'primary infected' animals was collected by gently scraping the mucosal surface with a glass microscope slide. Mucus scrapings were homogenized with an equal weight of phosphate-buffered saline (0·05 M PBS, pH 7·3, 3 mM Na azide) using an Ultra-turrax homogenizer (13000 r.p.m., 3 × 1 min). The homogenates were centrifuged at 20000 ***g*** for 30 min. The supernatant fraction was removed and stored at −70 °C. To isolate the immunoglobulins, the supernatant fraction was treated with Protein G–agarose beads (Roche). Mucus (1 ml) was centrifuged (14000 ***g***, 4 °C, 30 min) to remove the debris. Then 200 μl of starting buffer (20 mM NaH_2PO_4, pH 7·0) were added to the supernatant fraction to ensure that the pH of the sample stayed neutral. After equilibration of the sample (2 washes with starting buffer) 100 μl of protein G–agarose beads were added. The sample was placed on a rotor for 2 h at 4 °C to allow the binding of the Fc parts of the immunoglobulins to the beads. Supernatant was collected and saved together with the first 5 washes (400 μl of washing buffer/wash, 20 mM NaH_2PO_4, 150 mM NaCl, 2 mM EDTA, pH 7·0). The bound immunoglobulins were eluted with 400 μl of elution buffer (100 mM glycine, pH 2·7) until the OD of the elutions was zero. The fractions were immediately neutralized with 20% neutralization buffer (1 M Tris–HCl, pH 9·0). The supernatant/wash fraction was again treated with Protein G–agarose beads to ensure that all antibodies present in the mucus sample were collected. The treated mucus samples were pooled in 2 groups for each farm: the 'immunized' group and the 'primary infected' group (Table 1).

Antibody secreting cell probes (ASC-probes) collection

ACS-probes were collected from animals of farm 3 ($n = 13$). Antibody-secreting cell probes (ASC-probes) designate the supernatant of a lymph node cell culture that was prepared with the technique originally described by Meeusen & Brandon (1994*a*, *b*). In short, abomasal lymph nodes were collected at necropsy and transported in cold PBS + 1% penicillin–streptomycin. Lymphocytes were harvested by cutting and teasing the nodes in 5 ml of RPMI medium (Gibco BRL), washed in RPMI medium and centrifuged (1000 ***g***, 10 min, 4 °C). The red blood cells were lysed by adding 20 ml of lysis solution (2% Tris, pH 7·65, 0·8% NH_4Cl), for 10 min with gentle shaking. Twenty ml of RPMI containing 1% penicillin–streptomycin and 2% horse serum was used to wash the cells 3 times. Cells were resuspended to a final concentration of 5×10^6 cells/ml in culture medium (RPMI supplemented with 20% horse serum, 1% penicillin–streptomycin, 1% sodium pyruvate, 1% non-essential amino acids, 1% kanamycin, 0·1% gentamycin and 0·035% β-mercaptoethanol). Culture flasks containing 50 ml of cell suspension were incubated at 37 °C in an atmosphere of 5% CO_2 in air without stimulation. After 3 days, the cells were removed by centrifugation (1000 ***g***, 10 min) and 400 ml of supernatant per animal were collected. The supernatant (ASC-probes) was concentrated 10 times in a SpeedVac

Table 1. Pools of antibodies used to screen Western blots

Farm	Mucus antibodies immunized	Mucus antibodies primary infected	ASC antibodies immunized	ASC antibodies primary infected
Farm 1	Pool 1 ($n = 4$)	Pool 2 ($n = 6$)	—	—
Farm 2	Pool 3 ($n = 6$)	Pool 4 ($n = 6$)	—	—
Farm 3	Pool 5 ($n = 7$)	Pool 6 ($n = 6$)	Pool 7 ($n = 7$)	Pool 8 ($n = 6$)

and pools of antibodies both from the 'immunized' animals and the 'primary infected' animals were made for screening Western blots and cDNA libraries (Table 1).

Somatic worm extracts and excretory/secretory (E/S) products

Somatic extract was made by grinding the larval L_3, L_4 and adult worms in a mortar under liquid nitrogen and resuspending them in PBST (0·05 M PBS, 0·05% Tween-20). Insoluble fragments were spun down (25000 ***g***, 4 °C) and the clear supernatant fractions were filter sterilized (0·2 μm filter, Costar).

E/S products from L_3, L_4 and adult worms were prepared as described previously (Geldhof *et al.* 2000).

SDS-PAGE and immunoblotting

SDS-PAGE was performed with somatic extracts from L_3, L_4 and adult worms as well as with E/S products from the different life-stages, using a 4% stacking gel and a 10% running acrylamide gel (Laemmli, 1970). Ten μg of somatic extracts and E/S products were separated under reducing conditions. Samples were mixed with sample buffer and boiled for 5 min. For size estimation, high and low molecular weight protein standards (Bio-Rad) were electrophoresed simultaneously.

After electrophoresis, proteins were transferred to a polyvinyldine difluoride (PVDF) membrane (Immobilon®, Millipore) by semi-dry electrophoretic transfer at 250 mA for 2 h. Non-specific binding was blocked with 10% horse serum (HS) in PBST for 30 min. The blots were washed with PBST (3 × 10 min) and incubated overnight at 4 °C with concentrated ASC-probes or purified mucus IgG antibodies in PBST. Subsequently, the blots were washed in PBST (3 × 10 min) and developed with an indirect immunoperoxidase method using rabbit anti-bovine-IgG (H+L) (Jackson ImmunoResearch, Laboratories Inc., diluted 1:8000 in PBST with 2% HS) and horseradish peroxidase conjugated goat anti-rabbit (Sigma, diluted 1:1000 in PBST). 3′,3′ diaminobenzidine tetrachloride in PBS containing 0·02% (v/v) H_2O_2 was added to visualize the bound proteins. After incubation, the blots were washed in PBST and air-dried.

cDNA library screening

O. ostertagi L_3, L_4 and adult cDNA libraries were constructed in λgt11 phage, propagated on Y1090r$^-$ cells and plated by standard methods (Sambrook, Fritsch & Maniatis, 1989). Approximately 100000 plaques of all 3 libraries were screened with ASC-probes and mucus antibodies. All plaques were first screened with a pool of antibodies of 'immunized' animals from all 3 farms. All positive plaques were rescreened until a single plaque could be isolated. These positive plaques were rescreened with the antibody pool from 'primary infected' animals from all 3 farms. The plaques that were exclusively recognized by the antibodies from the 'immune' animals were retained, resuspended in 200 μl of sterile SM buffer (50 mM Tris–HCl, pH 7·5, 100 mM NaCl, 10 mM $MgSO_4$) and stored at 4 °C with a drop of chloroform. The others were designated false positives due to cross-recognition of the antibodies from the 'primary infected' animals.

The inserts were amplified by PCR reaction with universal λgt11 primers and the amplicon was gel-purified with a gel purification kit (Qiagen). The cDNA fragment was subcloned into pGEM-T vector (Promega) and transformed into DH5α *Escherichia coli* cells. Following blue-white screening (IPTG/X-gal) and PCR with SP6 and T7 vector primers, recombinant clones were selected and plasmid DNA was isolated using the Qiagen plasmid isolation kit. The nucleotide sequence of the cDNA clones was determined by the dideoxy chain terminator method using fluorescent BigDyeTM terminators in a 377 automated DNA sequencer (PE Biosystems). Assembly and analysis of nucleotide and deduced amino acid sequences were performed using the DNASTAR software program. Database searches were performed using the BLAST server (NCBI, EMBL, WU-blast2).

RESULTS

Immunoscreening of Western blots with local antibody probes

Screening Western blots of somatic extracts from L_3, L_4 and adult *O. ostertagi* with pools of antibodies from 'immunized' animals and 'primary infected' animals revealed a difference in recognition pattern.

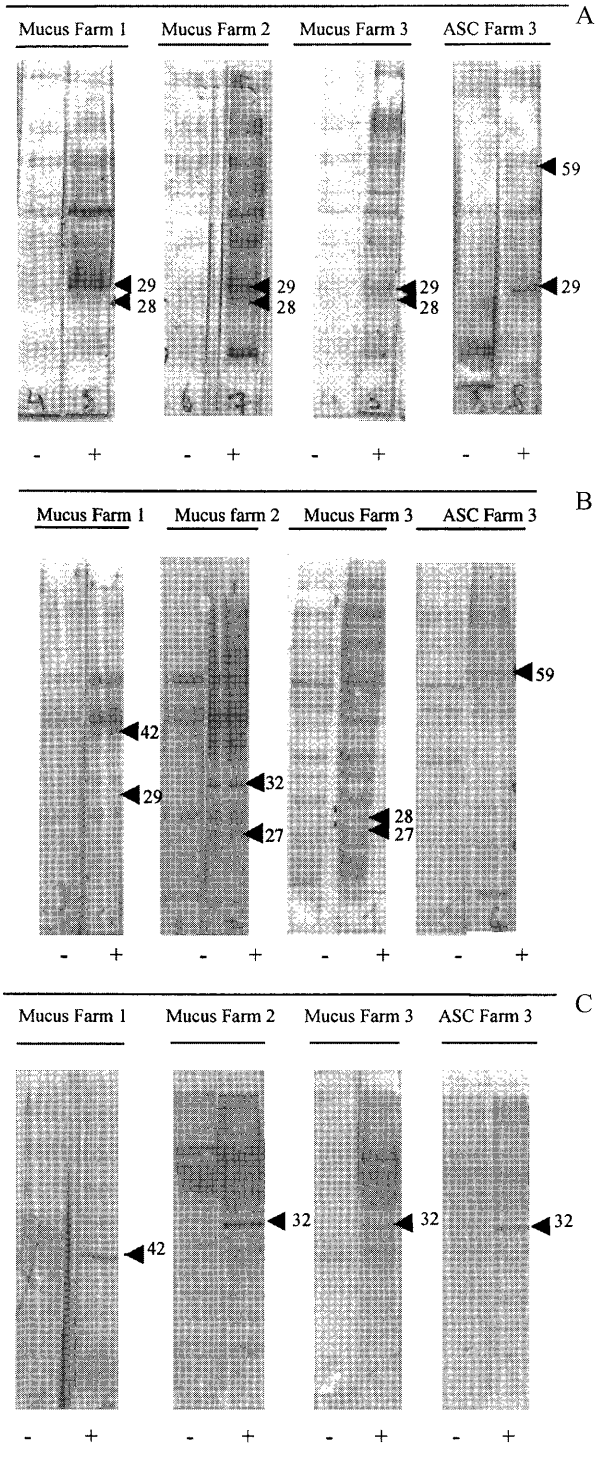

Fig. 1. Western blots of (A) L3, (B) L4 and (C) adult worm extracts immunoscreened with mucus antibodies and ASC antibodies from antibody pool of 'immunized' animals (+) and 'primary infected' animals (−).

These differences were clearest in the larval stages of the worm where proteins situated in the region of 27–29 kDa were specifically recognized by mucus antibodies from 'immunized' animals (Fig. 1A, B). Lymph node ASC antibodies from the 'immunized' animals recognized a L_3, L_4 59 kDa antigen (Fig. 1A, B). Mucus antibodies from farm 1 recognized an antigen of approximately 42 kDa (Fig. 1B, C) in the

Table 2. List of cDNAs from the expression libraries screened with local antibody probes, homologies and accession numbers

Name of clone	No. of clones	Homology	Closest species	Percentage homology	Library	Antibody probe	GenBank accession no.	Insert size (bp)
Metal I	3	Metalloprotease I	*A. caninum*	70	Adult, L4	Mucus	AJ419180	1238
Metal II	2	Metalloprotease II	*A. caninum*	67	L4	Supernatant	AJ420009	325
Metal III	1	Metalloprotease III	*A. caninum*	62	Adult	Supernatant	AJ426428	708
Api gene	3	Aspartyl Protease Inhibitor	*P. tenuis*	75	Adult, L3	Supernatant	AJ418047	900
ASP gene	2	*Ancylostoma* Secreted Protein-1	*A. duodenale*	51	Adult	Mucus	AJ310812	300
SXC1	2	Six Cysteine Motif	*O. ostertagi*	88	Adult	Supernatant	AJ427869	599
Col gene	2	Collagen	*C. elegans*	60	L4	Supernatant	AJ420010	287
CystBS gene	1	Cystathionine β-synthase	*C. elegans*	68	L4	Mucus	AJ419179	561
CytoC gene	1	Cytochrome c-oxidase	*A. suum*	77	Adult	Mucus	AJ426450	384
L4SNO.o gene	1	O.o partial mRNA for hypothetical protein	*C. elegans*	51	L4	Supernatant	AJ420011	557
Oo L4 01D10	1	OoL4SKPL cDNA clone	*H. contortus*	99	L3	Supernatant	BG734056	589
Oo L4 02D09	4	OoL4SKPL cDNA clone	*O. ostertagi*	96	L3	Mucus/Supernatant	BG734132	527
OoL3	1	OoL3EST clone	*H. contortus*	93	L3	Supernatant	AJ426451	424
20 kDa E/S antigen	1	20 kDa ES antigen	*O. ostertagi*	86	L3	Mucus	CAC44259	568
L3 unknown	1	L3 unknown protein	None	—	L3	Mucus	None	350

L_4 and the adult stage. In the adult stage, mucus antibodies and ASC antibodies from 'immunized' animals specifically detected a protein of 32 kDa (Fig. 1C). Recognition patterns were very similar within pools from all 3 farms. Western blots of E/S material from L_3, L_4 and adult *O. ostertagi* revealed no clear difference in recognition pattern between the 'immunized' antibody pools and the 'primary infected' antibody pools from the 3 farms (data not shown).

Immunoscreening of cDNA libraries with local antibody probes

In total, 35 positive plaques were isolated, 18 with ASC antibody probes and 17 with mucus antibodies, all of which contained inserts. Four clones could not be amplified by PCR with the λgt11 primers. Submission of the sequence data to the databases showed that 5 of the clones contained a non-relevant vector-insert. The remaining 26 clones represented 15 different proteins of which 6 showed no homology with a known functional protein. Six clones contained a metalloprotease homologue, 3 clones an aspartyl protease inhibitor homologue, 2 clones an *Ancylostoma* secreted protein-1 homologue, 2 clones contained 6 cysteine motifs, 2 clones were homologues of collagen, 1 of cytochrome c-oxidase, 1 of cystathionine β-synthase, 8 clones contained an insert with homology to nematode proteins with no specified function and 1 clone contained an insert without any homology. More details about the isolated clones concerning the homology of the clones with other species, the source of local antibodies used, the nematode life-stage they were found in, the insert size and the accession numbers of the cDNA, are listed in Table 2.

DISCUSSION

In the present study we used local antibody probes, collected from the draining lymph nodes and the abomasal mucus to screen Western blots and expression libraries to identify possible protective antigens.

Previously, the ASC-probe technique has only been used in pigs (Jungersen *et al.* 2001), and sheep (Meeusen & Brandon, 1994*a*, *b*; Bowles, Brandon & Meeusen, 1995) to identify specific proteins with protective properties against *Ascaris suum*, *H. contortus* and *Taenia hydatigena*. The use of abomasal mucus as a source of local antibodies to screen blots has, until now, not been extensively used. However, Stear *et al.* (1995) and Claerebout *et al.* (1999) suggested an important role for abomasal mucus antibodies in the regulation of fecundity of respectively *T. circumcincta* in sheep and *O. ostertagi* in cattle.

The present results validate the usefulness of the abomasal lymph nodes and mucus as a source of local antibodies for the detection of possible protective antigens of *O. ostertagi* in cattle. With these local antibody probes, it was possible to see a difference in recognition pattern on Western blot from parasite extracts between 'immunized' animals and 'primary infected' animals. Primary infected animals, killed 3 weeks after challenge infection, were used as negative controls. Because resistance to reinfection is only acquired after several months of infection (Gasbarre, 1997) antigens that are already recognized after 3 weeks of infection are most likely not specific targets of a protective immune response against *Ostertagia*. Due to paucity of the antibody samples, the Western blots were screened with 6 pools of antibodies ('immunized' and 'primary infected' from 3 different farms) instead of individual antibody probes. We can therefore not exclude the possibility that the difference in recognition pattern between the primary infected calves and the immunized calves was due to 1 individual in either group. However, the similarity in recognition pattern between the pools of the 3 different farms makes this highly unlikely.

The immunoscreening of the cDNA expression libraries with 1 pool of mucus/ASC 'immune' antibodies and 1 pool of 'primary infected' antibodies resulted in the isolation of 35 immunoreactive cDNA clones.

Metalloproteases

Proteolytic enzymes have been identified as drug targets and vaccine candidates in a variety of disease systems (Knox, 2000; Geldhof *et al.* 2000, 2002). In *H. contortus*, a highly protective gut membrane protein is an aminopeptidase (Munn *et al.* 1997) and a component of the protective gut glycoprotein complex H-gal-GP has been identified as a metallopeptidase (MEP1) (Redmond *et al.* 1997). A Blast search of GenBank using our metalloprotease predicted amino acid sequences indicated significant homology to members of a family of zinc metalloproteases called the astacins (Bond & Beynon, 1995). Members of the astacin family have a wide range of functions (Zhan *et al.* 2002) including digestion, tissue penetration (Hotez *et al.* 1990; Geldhof *et al.* 2000) hatching (Hawdon *et al.* 1995), growth-factor processing and pattern formation in embryos (Blelloch & Kimble, 1999) and immunomodulation (Culley *et al.* 2000).

Aspartyl protease inhibitor

Specific protein inhibitors of aspartyl proteinases have been isolated from parasites such as *Acanthocheilonema viteae* (Willenbücher, Höfle & Lucius, 1993), *Onchocerca volvulus* (Tume *et al.* 1997), *Dirofilaria immitis* (Frank *et al.* 1998), *A. suum* (Kageyama, 1998) and *Brugia malayi* (Maizels, Blaxter & Scott, 2001). Their physiological role is not known. They might protect the parasite from

peptic digestion when it migrates through the stomach. However, the presence of related pepsin inhibitor-like proteins in filarial parasites in lymphoid and connective tissue suggests that they also can have other functions, like inhibitory activity against cathepsin E playing a role in interference with the host immune system (Bennet *et al.* 1992). Protease inhibitors are also important in the control of endogenous proteinases involved in parasite development (Lustigman *et al.* 1992).

Ancylostoma *Secreted Protein-1*

ASP-1 is a 42 kDa protein, made up of 2 distinct but related domains, that is specifically released by the hookworm larvae in response to host signals (Hawdon *et al.* 1996). A critical and central role for ASP-1 in the transition from the external environment to parasitism was suggested and interference with its function might prevent infection with hookworm L3 and subsequent diseases, making ASP-1 a promising vaccine candidate (Hotez, Hawdon & Cappello, 1996; Ghosh, Hawdon & Hotez, 1996). The *O. ostertagi* homologue of the *Ancylostoma* secreted protein was, however, found in the adult stage. Also in the EST datasets from adult *Necator americanus* (Blaxter, 2000; Daub *et al.* 2000), *B. malayi*, *O. volvulus* (Blaxter *et al.* 1996) and *H. contortus* (Schallig *et al.* 1997) single-domain ASP homologues were found.

Six Cysteine Motif

The SXC domain (Blaxter, 1998), also termed NC6 (Gems *et al.* 1995), was first identified in surface coat proteins of the parasitic ascarid *Toxocara canis* (Maizels, Tetteh & Loukas, 2000; Loukas *et al.* 2000) and *C. elegans*. SXC domains have also been identified in other nematodes such as *Ascaris*, *Brugia*, *Trichuris muris* and *Necator* (Daub *et al.* 2000). The function of the motif is not known but it is suggested that it acts as a signalling ligand (Blaxter, 1998) or that it is involved in protein–protein interactions, particularly those associated with nematode surfaces.

Collagen

Studies on the free-living nematode *C. elegans* have demonstrated that expression of different collagen genes peaks at different times of the inter-moult period and that the collagen genes expressed at each moult may vary (Johnstone & Barry, 1996; Johnstone, 2000). In nematodes, collagen is involved in the formation of 2 distinct but essential structures, the basement membrane and the cuticle.

Analogous to the structure of collagen (Ramachandran, 1967), our sequences show several small blocks of Gly-Ala-Pro flanked by non-Gly-X-Y amino and carboxy terminal domains (AJ420010).

Cystathionine β-synthase

Cystathionine β-synthase is a multi-functional enzyme that plays a central role in cystathionine synthesis (Walker & Barrett, 1997) during the sulphur amino acid metabolism. The detoxification of thiol groups by conjugation with cysteine and by H_2S production is catalysed by cystathionine β-synthase and ensures protection against oxidative damage.

Cytochrome c-oxidase

Cytochrome c-oxidase is a protein complex that can carry protons from the intermembrane face of the mitochondria to the cytosol for oxygen reduction and that can catalyse ATP production. We found a cytochrome c-oxidase homologue of *O. ostertagi* in the adult stage. Adult *O. ostertagi* reside in the lumen at the surface of the host's abomasum where the oxygen tension is low. Like all other parasitic helminths, they exploit a unique mitochondrial respiratory chain as an adaptation to the microaerobic environment (Saz, 1981; Tielens, 1994). The function of the cytochrome c-oxidase in a completely anaerobic environment is not yet clear.

Homologues of unknown genes

Three clones (AJ420011, BG734056, AJ426451) have significant similarity to 'hypothetical genes' predicted by the *C. elegans* genome-sequencing project (*C. elegans* Genome Sequencing Consortium, 1998). These hypothetical genes are predicted based on coding potential, base composition and splicing predictions. Six clones showed significant similarities to known parasite nematode sequences but with no assigned function. Further investigation and localization must be carried out to reveal more details of these proteins.

Although several proteins described above are E/S proteins (metalloproteases, ASP-1 homologue), screening of Western blots of E/S surprisingly did not give a clear difference in recognition of antigens. Possible reasons for this can be the disadvantage of separating proteins with one-dimensional gels or the immunoreactivity of the glycosylations of proteins, which made it impossible to identify clear bands that were exclusively recognized by immunized animals.

Using monospecific antibodies, affinity purified against antigens expressed by the selected plaques, attempts were made to localize the cDNA encoded antigens on Western blot. Unfortunately it was not possible to link the sequences of the cDNAs with the previously identified proteins on Western blots. Cross-reaction of the monospecific antibodies with *E. coli* proteins on the plates on one hand and with extract and E/S antigens on the blots on the other, made it impossible to differentiate one specific band.

Also, the low titre of the antibodies in the mucus as well as in the supernatant produced very faint bands on the blots, and adding more monospecific antibody only increased the non-specific reactions.

In summary, we showed that it is possible to identify antigens that are specifically and exclusively recognized by local antibodies from mucus and ASC probes from 'immunized' animals. This fact alone makes them already interesting vaccine candidates. In addition, we identified several antigens of which preliminary experiments in other host–parasite systems have shown that they have potentially protective capacity (metalloprotease), immunodominant and diagnostic properties (aspartyl protease inhibitor) (Peanasky *et al.* 1987) or functional and physiological importance for the parasite (collagen, cystathionine β-synthase) (Walker & Barrett, 1997).

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